

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C07C 271/22, 323/60</b> <b>C07K 5/06, 5/08</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 91/09837</b> <b>(43) International Publication Date:</b> 11 July 1991 (11.07.91)
<b>(21) International Application Number:</b> PCT/AU90/00599 <b>(22) International Filing Date:</b> 19 December 1990 (19.12.90) <b>(30) Priority data:</b> PJ 8035 22 December 1989 (22.12.89) AU <b>(71) Applicant (for all designated States except US):</b> COMMON-WEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; 14 Limestone Avenue, Campbell, ACT 2601 (AU). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only) :</b> WHITTAKER, Robert, George [AU/AU]; 23 Ramsay Avenue, West Pymble, NSW 2073 (AU). <b>(74) Agent:</b> F. B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> AMINO ACIDS, PEPTIDES OR DERIVATIVES THEREOF COUPLED TO FATS  <b>(57) Abstract</b> <p>The present invention provides novel compounds in which peptides, amino acids or derivatives thereof are bound to other molecules, in particular fatty acids, which facilitate the use of such peptides, amino acids or derivatives thereof. The basic structure of the compounds of the present invention are an amino acid, peptide or derivative thereof linked to 1 to 3 fatty acid molecules via a tromethamine derivative. Alternatively, the amino acid, peptide or derivative thereof may be linked to a fatty acid by an ethanolamine derivative.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

- 1 -

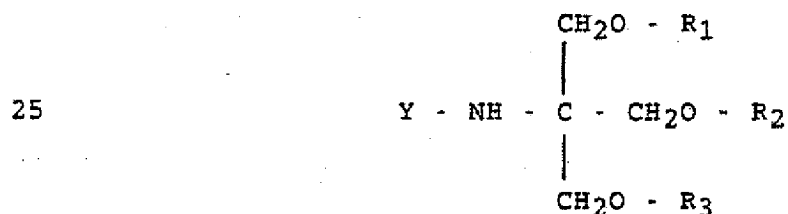
Amino acids, peptides or derivatives thereof coupled to fats

The present invention relates to novel compounds in which peptides, amino acids or derivatives thereof are bound to other molecules which facilitate the use of such peptides, amino acids or derivatives thereof.

In recent years there have been substantial advances made in the area of developing biological reactive peptides and peptide based compounds. One of the main difficulties encountered in the use of such compounds is the absence of an effective delivery system. It has been shown that antibodies raised against small peptides are active against large proteins (e.g. foot and mouth virus), however, such small peptides are rarely antigenic in the absence of an appropriate adjuvant.

The present inventor has discovered a novel means by which peptides and/or amino acid(s) and peptide derivatives may be linked to other molecules which may facilitate the therapeutic use of such peptides, amino acids or derivatives thereof. In addition, the present inventor has produced novel compositions having these characteristics.

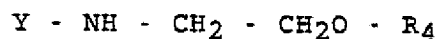
Accordingly, in a first aspect the present invention consists in a compound of the following formula:-



in which Y represents an amino acid, peptide or derivative thereof and  $\text{R}_1$ ,  $\text{R}_2$  and  $\text{R}_3$  are the same or different and are either hydrogen or fatty acids.

- 2 -

In a second aspect the present invention consists in a compound of the following formula:-



in which Y represents an amino acid, peptide or derivative thereof and R<sub>4</sub> represents hydrogen or a fatty acid.

As will be recognised by persons skilled in the art the compound of the first aspect of the present invention consists of an amino acid, peptide or derivative thereof linked to a tromethamine derivative to which is optionally linked a fatty acid(s). Similarly, the compound of the second aspect can be recognised as an amino acid, peptide or derivative thereof linked to an ethanolamine derivative to which is optionally linked a fatty acid.

In a preferred embodiment of the first aspect of the present invention, each of R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are a fatty acid, and more particularly, are each the same fatty acid. It is presently preferred that the fatty acid has a carbon chain of 3 to 18 carbon atoms, more preferably 10 to 18 carbon atoms, most preferably 16 carbon atoms. In the same manner, it is preferred that R<sub>4</sub> is a fatty acid having a carbon chain of 3 to 18 carbon atoms, more preferably 10 to 18 carbon atoms, and most preferably 16 carbon atoms.

In a further preferred embodiment of the first and second aspects of the present invention Y is cysteine. It is believed that cysteine in this position would be used to create a generic reagent(s) that could be coupled to free cysteines in proteins, peptides or derivatives thereof using standard cross linking reagents.

The novel compounds of the present invention enable alternative presentation of an amino acid, peptide or derivative thereof due to its position of attachment of fatty acids. Some of the potential uses of the novel

- 3 -

compounds of the present invention are:-

1. Immune enhancing affects of fatty acid-peptide conjugates. A large amount of research has been conducted into the potentiation of the immune response using fatty acid-peptide complexes. It is known that the presence of fatty acids does lead to an enhanced immune response to the peptide (Baschang, 1989, Tetrahedron, 45:6331-6360; Floc'h et al, 1984, Drugs Future, 9: 763-776; Jung et al, 1985 Angewandte Chemie Int. Ed. Eng. 24 (10) pp872-873.
2. Slow release delivery of peptides. Liposomes have been used as delivery and slow release vectors. (Moroder and Muriol, 1989, in Peptides Chemistry, Structure and Function, J.E. Rivier & G.R. Marshall Eds pp 811-812). Attaching fatty acids to peptides would help their incorporation into liposomes as well as be a method of incorporating peptides into "oily depots". (US 4829142; Gregoriadis, 1989, in Targeting of Drugs : Implications in Medicine, F.H.D. Roerdinl & AM Kroon Eds pp 1-29).
3. Altering the mechanism of action. (De Vrije et al 1990, J. Mol Microbiol., 4 : 143-150). Fatty acids attached to peptide hormones etc. could alter their biological activity e.g. by prolonging biological half life.
4. Fats are absorbed intact from the digestive system. It is possible that a peptide/fatty acid complex could be administered orally to deliver bioactive peptides and/or antigenic peptides.
5. Tromethamine (TRIS) in general has a variety of industrial uses including an emulsifying agent for cosmetic creams and lotions. Therapeutically used as an alkalizer (Merch Index, Vol 11, entry 1536). It is possible that some of these uses could be improved by the addition of amino acids or peptides optionally

- 4 -

with fatty acids (Molinero et al 1988, JAOCS, 65: 975-978).

The present inventors envisage that by attaching peptides or peptide derivatives by their C-terminal to either TRIS- tripalmitate (or other mono, di or tri constructs with various fatty acids) or amino acid-TRIS-fatty acid constructs it will be possible to facilitate interaction of the peptides-fats with a variety of lipid structures (both natural and artificial) e.g. liposomes of various types, cell walls, membranes etc. (ethanolamine and similar compounds may be used in place of TRIS). By this manner alternative methods can be derived for the delivery and in vivo presentation of peptides and for modifying their mode of action and antigenicity. For example, the use of synthetic antigens in various veterinary and human pharmacology still sets the very important problem of finding a suitable and inexpensive carrier devoid of side effects. Carriers used in animal trials, such as keyhole limpet haemocyanin (KLH), bovine serum albumin, ovalbumin and the tetanus toxoid are generally unsuitable for human application due to side effects such as immunogenicity. In addition some of them (e.g. KLH) have the additional problem of being very expensive.

Potentially more acceptable synthetic substitutes have been investigated by a number of laboratories.

The full chemical synthesis of the N-terminal lipopeptide of the outer membrane of E.coli has been described by G. Jung and his collaborators (Bessler et al 1984, Biochem. Biophys. Res. Comm. 121, pp55-61 and Jung 1988 "Low Molecular Weight Lipopeptide Carrier-Adjuvant Systems for Immunisations: Developments and Results" in Peptide Chemistry T. Shiba and S. Sakakibara Ed. p.751-758) and intensive studies of the various peptide conjugates have been described. These compounds consist

- 5 -

of three fatty acid molecules linked to S-(dihydroxypropyl) cysteine attached to a tetrapeptide of the sequence Ser-Ser-Asn-Ala. This type of construct and analogues of it have mitogenic activity and can be used to anchor a variety of biological agents in lipid membranes.

A completely synthetic, low molecular weight vaccine against foot and mouth disease virus (FMDV) was developed in this manner (Weismuller et al 1989, Vaccine 1, pp29-33). A sequential epitope of FMDV surface glycoprotein (135-154) coupled to the tripalmitoyl-S-glyceryl-cysteinyl-serine-serine moiety induced long lasting high protection against foot and mouth disease after a single administration without any adjuvant or carrier. Similarly, a vaccine against influenza virus has been prepared (Deres et al 1989, Nature 342, pp561-564).

Other biological effects noted for this type of construct include the induction of cytotoxic T lymphocytes (Deres et al, as above), the release of cytokines such as interferon and tumour necrosis factor alpha from macrophages (Hauschildt et al 1990, Eur. J. Immunol. 20, pp63-68) and the activation of neutrophils (Seifert et al 1990, Biochem. J. 267, pp795-802).

A second novel oligopeptide delivery system for poorly absorbed peptides and drugs has been described by Toth et al 1989 ("A Novel Oligopeptide Delivery System for Poorly Absorbed Peptides and Drugs" in Peptides, Chemistry, Structure and Function, pages 1078-1079). This system is based on the development of synthetic, non natural amino acids with long alkyl side-chains; so called "fatty amino acids".

The covalent attachment of these compounds to various drugs has been reported to have increased oral absorption as well as overcoming the limited passage of e.g. GABA across the blood-brain barrier.

TRIS and ethanolamine seem to be ideal bi-functional

- 6 -

molecules to join peptides (via their carboxyl group to the NH<sub>2</sub> group of TRIS or ethanolamine) and fats (via ester linkage to the hydroxyl group(s) of TRIS or ethanolamine). To demonstrate the feasibility of this approach several amino acids and peptides have been reacted with TRIS and the products characterised. Four of these, Z-Ala-TRIS, Z-Gly TRIS, Z-Leu-TRIS and BOC-Ala-Ile-Phe-TRIS were successfully palmitoylated and purified. As well as constructs of 2-Ala-TRIS with sloster chain fatty acids. After removal of the N-protecting group, the free amino terminal would be available to react with other amino acids or peptides either by enzymatic procedures or by traditional methods of peptide chemistry.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following examples and Figures in which:-

Fig. 1 shows a time profile of Z-Ala-OMe plus TRIS incubated at 60°C, pH8 in 60% DMF (—□—) percentage efficiency; (—◆—) percentage substrate used after 7 days;

Fig. 2 shows substrate preference; 60°C incubation for 18 hours, % efficiency (▨) and substrate used (■) vary with ester type and the nature of the amino terminal blocking group;

Fig. 3 shows temperature profile: BZ-Ala-OMe plus TRIS; 60% DMF pH 8.0 18 hours (—◆—) percentage substrate used; (—□—) percentage efficiency;

Fig. 4 shows pH profile Z-Ala-TRIS; 60% DMF incubated 2 days 60°C (—□—) efficiency; (—◆—) percentage substrate used;

Fig. 5 DMF profile Z-Ala-TRIS; pH 9 incubated 2 days 60°C (—□—) efficiency; (—◆—) percentage substrate used;



- 7 -

Fig. 6 shows  $^3\text{H}$ -thymidine uptake in RD10 cells  
 (—□—) ATP3; (—◆—) ZATP3; and

Fig. 7 shows  $^3\text{H}$ -thymidine uptake in U937 cells  
 (—□—) ATP3; (—◆—) ZATP3.

## 5 MATERIALS AND METHODS

Abbreviations:-

- TLC Thin layer chromatography
- HPLC High Performance Liquid Chromatography
- NMR Nuclear Magnetic Resonance
- 10 GLC Gas-liquid chromatography
- DCM Dichloromethane
- DCC Dicyclohexylcarbodiimide
- DCU Dicyclohexylurea
- DMAP Dimethylaminopyridine
- 15 DMF Dimethyl formamide
- Z Benzyloxycarbonyl
- Bz Benzoyl
- BOC Tertiary butyloxycarbonyl
- TRIS Tromethamine
- 20 OMe, OEt, OBzl Methyl, Ethyl and Benzyl esters resp.
- Ala, Leu, Gly, The amino acids:- alanine, leucine,
- Ile, Phe, Tyr, glycine, isoleucine, phenylalanine,
- Arg, Trp, Lys & tyrosine, arginine, tryptophan, lysine
- Val and valine respectively

## 25 A. CHEMICAL AND ANALYTICAL METHODS

All solvents were analytical reagents and those used for HPLC were especially purified for liquid chromatography.

### THIN LAYER CHROMATOGRAPHY

- 30 i) Plates:-
  - a) Pre-coated TLC plates, Silica gel 60 (without fluorescence indicator, Merck 5721) 20x20 cm, 0.25 mm thickness.
  - b) TLC aluminium sheets, silica gel coated (with fluorescence indicator) DC Alufolien Kieselgel 60
- 35

- 8 -

20x20 cm, 0.2 mm thickness, Merck 5554.

c) TLC aluminium sheets, pre-coated with cellulose (20x20 cm, 0.1 mm thickness, Merck 5552).

ii) Detection of compounds:-

- 5 a) Ninhydrin 0.1% (Merck ART, 6758)  
b) 2,4 dichlorofluorescein, 0.2% in ethanol  
c) iodine atmosphere

#### SOLVENT SYSTEMS

- |       |  |              |
|-------|--|--------------|
| a.    | chloroform/methanol/water              | 13:5:0.8*    |
| 10 b. | chloroform/methanol/ammonia 17%        | 70:35:10*    |
| c.    | chloroform, saturated*                 |              |
| d.    | ether, saturated*                      |              |
| e.    | petroleum ether/ether/acetic acid      | 70:30:1*     |
| f.    | butanol/acetic acid/ water/acetic acid | 5: 2:3*      |
| 15 g. | petroleum ether/ether/acetic acid      | 50:50:1*     |
| h.    | ethyl acetate saturated with water*    |              |
| i.    | ethyl acetate/chloroform/ammonia 17%   | 98:6:1*      |
| j.    | chloroform/methanol                    | 9:1*#        |
| k.    | chloroform/methanol                    | 8:2*#        |
| 20 l. | ether/benzene/ethanol/acetic acid      | 40:50:2:0.2* |

Rf values were determined at 20°C

\*TLC; # Column

#### COLUMN CHROMATOGRAPHY

25 Three methods were used for separation of palmitoylated (or other fatty acid acylated) TRIS constructs:-

i) LH SEPHADEX

SEPHADEX LH 20 (PHARMACIA, Fine Chemicals) was packed into glass columns 1x25 cm) and samples eluted with 30 chloroform/methanol mixtures at a rate of 1 ml/min. Fractions (5 ml) were collected and their contents analysed by TLC. Separation is by size.

ii) SILICA GEL COLUMN

35 Samples were loaded onto a silica gel column (BDH 60-120, size 2.6 x 30 cm) and eluted with chloroform/

- 9 -

methanol mixtures (9:1 or 8:2 as required) at a rate of 2 ml/min. The effluent was monitored at 260nm (ETP KORTEC, K95 UV monitor), 5 ml fractions collected and tested by TLC. Separation is by charge and hydrophobicity.

- 5   iii) FLORISIL COLUMN for separation of neutral lipids (a gift from Dr. Fogarthy, CSIRO Div. of Food Preservation). Chromatography was in glass columns of 12 gr acid washed FLORISIL and eluted with hexane/ethyl ether mixtures according to Carroll, K.K. and Serdarevich, B. (1967) in  
10 "Lipid Chromatography Analysis" p.205-237, Dekker, New York. The effluent was monitored by TLC.

#### ANALYSES

- i) Elemental analyses (CHN ratio) were carried out by the Analytical Unit of the Chemistry Department, Research  
15 School of Chemistry Australian National University GPO Box 4, Canberra.
- ii) <sup>13</sup>C and PROTON NMR SPECTRA were done by the CSIRO Division of Biomolecular Engineering, Parkville, Vic. 3052. The compounds of the present invention behaved as  
20 expected with a slow exchange of protons which are good indications of peptide bonds.
- iii) GLC analysis for fat content were done by Analchem Consultants Pty. Ltd.
- iv) HPLC. Analytical HPLC of the N-protected amino  
25 acid-TRIS compounds were carried out using Millipore Waters HPLC equipment 6000A series solvent delivery system, connected to a Lambda Max Spectrophotometer Model 480, an Automated Gradient controller and Model 746 Data Module. Analytical HPLC was performed on a 100 x 8 mm  
30 NovaPak C<sub>18</sub> reverse phase radial pack cartridge and eluted at a flow rate of 2-3 ml/min. with a triethylamine phosphate pH3/acetonitrile gradient. The eluted substances were detected spectrophotometrically at 254 nm. The program used was a 20-100(6)5'(2) where 20-100 is  
35 the % acetonitrile at the beginning and end; (6) is the

- 10 -

gradient program (6 is linear); 5' is time in minutes;  
(2) is the flow in ml/min.

#### Preparative HPLC

Separations were performed on a Millipore Waters  
5 Prep500 HPLC. The column was PrepPAK 500-C<sub>18</sub> (125A and  
55-105 micron particle size) and the eluant was various  
concentrations of aqueous ethanol at 100 ml/min. Some  
separations required the addition of acetic acid at 1 ml/L.  
PROTEASE TREATMENT OF Z-Ala-TRIS, Z-Gly-TRIS and

#### 10 Z-Leu-Arg-TRIS

Z-Gly-TRIS and Z-Ala-TRIS were incubated with papain,  
and Z-Leu-Arg-TRIS with trypsin, to test that the action  
of proteolytic enzymes could regenerate the starting  
compounds Z-Gly-OH, Z-Ala-OH and Z-Leu-Arg-OH respectively.

#### 15 i) Activation of Papain

50 ul of a suspension of papain (25 mg/ml, Sigma) was  
incubated with 20 mM aqueous solution of EDTA (50 ul); 1  
Molar solution of mercaptoethanol (25 ul); 50 mM  
triethylamine (25 ul); and water (100 ul) at 37°C for  
20 30 min.

#### ii) Digestion Examples

a) Papain. Z-Ala-TRIS in 50 mmolar phosphate buffer pH  
6.5 (650 ul), 20 mM ethylene-diamine-tetra-acetic  
acid (100 ul) and water to 1 ml. Activated papain  
25 (5mg/ml) was added to a final enzyme/substrate ratio  
of 1:50. The reaction was followed by HPLC on C<sub>18</sub>  
reverse phase columns and the hydrolysis product  
identified by co-chromatography with standards.  
Blanks were incubated as above using water instead of  
30 enzyme solution.

b) Trypsin. Z-Leu-Arg-TRIS was incubated at a  
concentration between 2 and 10 mg/ml in 0.2 M  
ammonium hydrogen carbonate. Freshly prepared TPCK  
trypsin (1 mg/ml in water) was added to the solution  
35 giving a final enzyme/substrate ratio of 1:50.

- 11 -

Samples were incubated at 37°C and aliquots examined by reverse phase HPLC. Additional amounts of enzyme were added as required.

#### RESULTS

5 i) Z-Ala-TRIS and Z-Gly-TRIS.

HPLC was used to monitor the progress of enzymatic hydrolysis. Both Z-Ala-TRIS and Z-Gly-TRIS were hydrolysed by papain, albeit very slowly. The main product was identical with Z-Ala-OH and Z-Gly-OH respectively by co-chromatography (i.e. spiking). This is a check that the type of linkage between the protected amino acid and TRIS is the same in the products made by method A and B (see below) and that it is most likely an amide bond. In addition we have the evidence from the NMR analysis of Z-Ala-TRIS (incubation method) that the bond is an amide linkage. Also, elemental and GLC analyses on fatty acid derivatives indicate three fat molecules per molecule of TRIS indicating the presence of three OH groups on the amino acid-TRIS derivative used in the esterification.

ii) Z-Leu-Arg-TRIS was hydrolyzed slowly with trypsin. The product of hydrolysis was shown to be identical with an authentic sample of Z-Leu-Arg-OH by co-chromatography on HPLC.

25 The amino acid-TRIS compounds were made by incubation of the N-protected amino acid esters and peptide esters with TRIS in 60% aqueous DMF at elevated temperature (see incubation method). A typical reaction time profile is shown in Figure 1, effect of Z versus BZ plus ester types are shown in Figure 2, temperature profile in Figure 3, pH profile in Figure 4 and DMF profile in Figure 5.

As additional proof that the amino acid to TRIS linkage was a true amide bond, Z-Ala-TRIS and Z-Gly-TRIS were prepared by both the "incubation procedure" and normal organic chemistry methods for comparison by TLC.

- 12 -

Tri-palmitoylated forms of these two compounds were also prepared and compared by TLC. In a recent work by Otvos et al on the solid phase synthesis of certain glycopeptides (Peptide Research, 1989, 2, 362) the observation was made that in coupling of amino acids to carbohydrates unprotected hydroxyl groups are mostly unaffected when the symmetrical anhydride method is used. Symmetrical anhydrides of Z-Ala-OH and Z-Gly-OH were prepared and reacted with large excess of TRIS. The major products were indistinguishable by HPLC, TLC and CHN ratio from Z-Ala-TRIS and Z-Gly-TRIS prepared by the incubation method. Accordingly, we are confident that this method will only interact with the NH<sub>2</sub>-group of TRIS and not any of the CH<sub>2</sub>OH groups. It is also to be noted that only one synthetic compound is evident during the incubation method.

#### EXAMPLES

##### 1. PREPARATION OF Z-AMINO ACID-TRIS AND ETHANOLAMINE COMPOUNDS

##### 20 METHOD A - CHEMICAL SYNTHESIS (SYMMETRICAL ANHYDRIDE METHOD)

##### EXAMPLE 1. Z-Ala-TRIS

The title compound was produced from Z-Ala-OH via the symmetrical anhydride procedure (Schussler, H., Zahn, H., Chem Ber. 95, p.1076 (1962) and Bodansky, M., and Bodansky, A. (1984) "The Practice of Peptide Synthesis". p.102, Springer-Verlag).

Z-Ala-OH (8g) in DCM (50ml) and a few drops of DMF was reacted with DCC(4.2g) to form the symmetrical anhydride. The DCU so formed was filtered off and the solvent removed in vacuo. The residue was dissolved in DMF (50 ml) and added to a solution of TRIS in DMF (10 g/80ml) and stirred at room temperature for 16 hours.

Purification of the title compound was by preparative HPLC which yielded an analytically pure white product.

- 13 -

Yield 2.90 g (49% theory); Rf 0.49 (solvent h) and 0.78 (solvent b), and Rt 5.19' on HPLC. Anal. calc. for  $C_{15}H_{22}N_2O_6$  : C55.20, H6.80, N8.58. Found C55.28, H6.87, N8.13. The compound had identical characteristics to that formed by the "Incubation Method" - Method B (see Example 3).

#### EXAMPLE 2. Z-Gly-TRIS

Z-Gly-TRIS was prepared in the same manner as Example 1 i.e. from Z-Gly-OH (7q) and purified by preparative HPLC. Yield 1.50 g (29% theory). The resulting Z-Gly-TRIS was chromatographically identical with the corresponding compound made by method B (see Example 4). Rf 0.36 (solvent h) and 0.66 (solvent b), and Rt 4.97' on HPLC. Anal. calc. for  $C_{14}H_{20}N_2O_6$  : C53.84, H6.45, N8.97. Found C54.53, H6.84, N8.85.

#### METHOD B. INCUBATION METHOD

#### EXAMPLE 3. Z-Ala-TRIS

5.04 g of Z-Ala-OMe (50 mmole) was added to 20.37 g. of TRIS (400 mmole) in DMF (252 ml) and water 168 ml) and incubated at 55°C at pH 9.0. The reaction was monitored by HPLC which showed that after four days the conversion to the title compound was complete with a 65% efficiency as determined by HPLC.

Purification by preparative HPLC gave an analytically pure Z-Ala-TRIS as a white powder. Rf 0.49 (solvent h) and 0.79 (solvent b). HPLC Rt was 5.19 minutes. Anal. calc. for  $C_{15}H_{22}N_2O_6$  : C55.20, H6.80, N8.58. Found C56.02, H7.03, N8.00. The structure was confirmed by NMR analysis.

#### EXAMPLE 4. Z-Gly-TRIS

10.02 g of Z-Gly-OBzl was added to 32.4 g of TRIS in DMF (401 ml) and water (267 ml) and incubated at 60°C at pH 9.0 for two days. Purification by preparative HPLC gave analytically pure Z-Gly-TRIS as a white powder. Yield 3.98 g (38.3% theory); Rf 0.36 (solvent h) and 0.66

- 14 -

(solvent b); HPLC Rt was 4.97'. Anal. calc. for  $C_{14}H_{20}N_2O_6$  : C53.84, H6.48, N8.97. Found C54.15, H6.56, N8.68. The structure was confirmed by NMR analysis.

EXAMPLE 5. Z-Leu-TRIS

5 Z-Leu-OMe (5g) and TRIS (16.2 g) in DMF (200 ml) and water (134 ml) was incubated at pH 8.0 and 55°C in the manner previously described (Example 3).

Purification by preparative HPLC gave 3.60 g (54% of theory) of analytically pure title product; Rf 0.62 (solvent h) and 0.25 (solvent i); HPLC Rt 6.37'.

EXAMPLE 6. Z-Leu-Arg-TRIS

Z-Leu-Arg-OMe (5.17 g) and TRIS (10.67 g) in a solution of DMF (132 ml) and water (88ml) was incubated at 55°C for seven days in the manner previously described. 15 The efficiency of the reaction was 55% by HPLC. Pure product was isolated by preparative HPLC. TLC Rf 0.70 (solvent f) and 0.02 (solvent b); Rt 5.37'.

EXAMPLE 7. BOC-Ala-Ile-Phe-TRIS

8.6 g of BOC-Ala-Ile-Phe-OMe was added to 20 g of 20 TRIS in DMF (240 ml) and water (160ml). The mixture was incubated at 55°C for four days (yield by HPLC 66%) and the title compound purified by preparative HPLC to give the analytically pure product (yield 1.4 g; 14% theory); Rf 0.50 (solvent h) and 0.85 (solvent b); HPLC Rt 6.55'. 25 Anal. calc. for  $C_{26}H_{45}N_4O_8$  : C57.65, H8.37, N10.34. Found C58.01, H8.12, N9.57.

EXAMPLE 8

Z-Ala-ETHANOLAMINE

Z-Ala-OEt (5.73 g) was incubated in DMF (273 ml) and 30 water (91ml) at 50°C with 91 ml ethanolamine at pH 11.

The reaction was monitored by HPLC and after twenty hours the formation of the title compound was complete with 82% efficiency by HPLC. Preparative HPLC yielded a chromatographically pure product (2.26 g; 38% theory); 35 Rf 0.50 (solvent h) and 0.82 (solvent b); HPLC Rt 5.10'.



- 15 -

EXAMPLE 9FORMATION OF AMINO ACID - TRIS COMPLEXES

- Comparative reactions were run using Z-Leu-OMe;  
 Z-Ala-OMe; Bz-Tyr-OEt; Z-Ala-OBzl; Z-Arg-OMe;  
 5 Z-Val-OMe and Bz-Arg-OMe. The efficiency of the reaction  
 of these compounds with TRIS and the rate at which the  
 reaction proceeded are set out in Tables 1 and 2,  
 respectively.

TABLE 1

10	<u>Substrate</u>	<u>Efficiency</u>	<u>Efficiency</u>	<u>Efficiency</u>
		<u>1 Day</u>	<u>3 Day</u>	<u>7 Day</u>
		<u>Incubation</u>	<u>Incubation</u>	<u>Incubation</u>
	Bz-Tyr-OEt	81%	79%	--
	Z-Leu-OMe	81%	76%	76%
15	Z-Ala-OMe	78%	77%	73%
	Z-Ala-OBzl	73%	71%	69%
	Z-Arg-OMe	72%	72%	67%
	Z-Val-OMe	66%	61%	--
	Bz-Arg-OMe	58%	75%	--

TABLE 2

20	<u>Substrate</u> <u>50mm</u>	<u>Rate (Used)</u>	<u>Rate (Used)</u>	<u>Rate (Used)</u>
		<u>1 Day</u>	<u>3 Day</u>	<u>7 Day</u>
		<u>Incubation</u>	<u>Incubation</u>	<u>Incubation</u>
	Bz-Arg-OMe	68.51%	86.12%	--
25	Z-Arg-OMe	62.30%	91.63%	100%
	Z-Ala-OMe	48.16%	83.87%	100%
	Z-Ala-OBzl	43.98%	86.53%	94.8%
	Z-Leu-OMe	24.24%	64.29%	85.28%
	Bz-Tyr-OEt	22.89%	52.15%	--
30	Z-Val-OMe	4.19%	22.40%	--

- 16 -

EXAMPLE 10FORMATION OF Z-LEU-ARG-TRP-TRIS

5.28 grams of Z-Leu-Arg-Trp-OMO was added to 7.6 grams of TRIS in 160 mls of solvent consisting of 60% DMF and 40% H<sub>2</sub>O. The reaction was allowed to proceed at 60° for four days at pH 10. Z-Leu-Arg-Trp-TRIS was recovered by preparative HPLC. Efficiency by HPLC - 43%. R<sub>f</sub> 0.72 (solvent f).

EXAMPLE 1110 FORMATION OF Bz-Tyr-Arg-Lys-TRIS

5.025 grams of Bz-Tyr-Arg-Lys-OE was added to 7.275 grams of TRIS in 150 mls. of solvent comprising 60% DMF and 40% H<sub>2</sub>O. The reaction was allowed to proceed at 60° for four days 50% yield by HPLC and Bz-Tyr-Arg-Lys-TRIS was recovered by preparative HPLC.

2. PREPARATION OF FATTY ACID COMPLEXESEXAMPLE 12Z-Ala-TRIS-TRIPALMITATE

A solution of palmitic acid (8.10 g) in chloroform (100 ml) was added to a stirred solution of Z-Ala-TRIS (2.75 g) in chloroform (60 ml) and DMF (15 ml) and the mixture cooled in ice. After dropwise addition of 6.50 g of DCC in chloroform (30 ml) followed by 0.4 g of DMAP in chloroform (10 ml), stirring was continued at 0°C for two hours and then at room temperature for 18 hours. The esterification reaction was monitored by TLC (solvent systems b, h, i and j). The resulting DCU was filtered off and the chloroform solution serially washed with water; 10% aqueous citric acid; water; 10% aqueous sodium hydrogen carbonate and water. The solution was dried over sodium sulphate and the solvent evaporated under reduced pressure. The crude product was purified by chromatography by three different methods:- LH Sephadex; silica gel and Florisil. All techniques gave analytically pure products that gave a single spot on TLC; R<sub>f</sub> 0.72

- 17 -

(solvent g) and Rf 0.29 (solvent e). Yields were 62.0%, 63.8% and 68.5% respectively. Anal. chem. for  $C_{63}H_{112}N_2O_9$  : C72.65, H10.84, N2.69. Found C72.58, H11.22, N2.31.

5 Fat (palmitic acid) content by GLC analysis was 70.1% indicating full esterification of all three hydroxyl groups of the Z-Ala-TRIS (Theory 68.7%). NMR results were consistent with the proposed structure. Products made with Z-Ala-TRIS prepared by both the chemical and  
10 incubation methods yielded analytically indistinguishable products.

#### EXAMPLE 13

##### Z-Gly-TRIS-TRIPALMITATE

0.94 g of Z-Gly-TRIS was reacted with 3.30 g of  
15 palmitic acid; 2.5 g of DCC and 0.12 g of DMAP (as per Example 12) to give a crude sample of the title product, 2.05 g, 67% Theory. Purification on LH Sephadex and silica gel gave analytically pure product in 43% and 45% yield respectively; TLC Rf 0.18 (solvent e) and 0.85  
20 (solvent l).

Fat (palmitic acid) content by GLC analysis was 70% indicating full esterification of all three hydroxyl groups of the Z-Gly-TRIS (Theory 69.6%). NMR results were consistent with the proposed structure. Products made  
25 with Z-Gly-TRIS prepared by both the chemical and incubation methods yielded analytically indistinguishable products.

#### EXAMPLE 14

##### Z-Leu-TRIS-TRIPALMITATE

30 1.10 g of Z-Leu-TRIS was reacted with 3.30 g of palmitic acid; 2.5 g of DCC and 0.12 g of DMAP (as per Example 12) to give a crude sample of the title product. Purification on a silica column yielded 1.78 g (54.9% Theory) of analytically pure product; TLC Rf 0.90  
35 (solvent g) and 0.87 (solvent k). Anal. calc. for

- 18 -

C<sub>67</sub>H<sub>121</sub>N<sub>2</sub>O<sub>9</sub> : C73.08, m H11.06, N2.58. Found  
C73.74, H11.52, N2.29. Fat content 66.0% (Theory 66.1%).

EXAMPLE 15Z-Ala-TRIS-MYRISTATE

- 5        Z-Ala-TRIS (326 mg) was reacted with myristic acid  
(914 mg) in chloroform in a similar manner to Example 10.  
DCC (825 mg) and DMAP (40 mg) in cold DCM (10ml) was added  
and stirred for two hours at 0°C followed by 16 hours at  
room temperature. Initial purification yielded 720 mg  
10 crude product. Precipitation from DCM/acetonitrile  
yielded 640 mg. (66.9% of the theoretical) of the title  
product. TLC gave a single spot of R<sub>f</sub> 0.63 (solvent g)  
and 0.29 (solvent e).

EXAMPLE 1615 Z-Ala-TRIS-TRILAURATE

- 326 mg of Z-Ala-TRIS and 801 mg lauric acid was  
reacted with 825 mg DCC and 45 mg DMAP in a manner similar  
to that described for Example 12. Evaporation of the  
solvent yielded 710 mg of crude title product. This was  
20 further purified on a Florisil column using the  
hexane-ethyl ether solvent mixtures as eluants. 420 mg  
(49.9% of theoretical) of the title compound was  
recovered; TLC R<sub>f</sub> 0.59 (solvent g) and 0.27 (solvent e).

EXAMPLE 1725 Z-Ala-TRIS-TRICAPRATE

- 326 mg of Z-Ala-TRIS and 464.7 mg of capric acid  
(n-decanoic acid) was reacted with 825 mg of DCC and 45 mg  
of DMAP in DCM in a manner described in Example 13.  
Purification on Florisil with hexane/ether and methanol  
30 gave chromatographically pure title product; yield 480 mg  
(61% Theory); R<sub>f</sub> 0.54 (solvent g) and 0.25 (solvent e).  
Anal. calc. for C<sub>45</sub>H<sub>76</sub>N<sub>2</sub>O<sub>9</sub> : C68.49, H9.71,  
N3.55. Found C68.96, H10.230, N3.18. Fat content by GLC  
was 63% (Theory 58.9%).

- 19 -

EXAMPLE 18Z-Ala-TRIS-MONOPALMITATE

700 mg of Z-Ala-TRIS was reacted with 500 mg palmitic acid and 410 mg DCC and 20 mg DMAP in the manner similar to that described in Example 12 except a 1.3 molar excess of reagents was used instead of the usual 4 molar excess to generate a construct with predominantly one fatty acid attached: some di and tri-palmitoylated product would also be expected. Yield of crude, mixed products 1.12 g (Theory 1.20 g).

Fractionation on Florisil gave three major products in the ratio of 0.27:1:1 with Rf's on solvent g of 0.73; 0.26 and 0.0 respectively. Fractions 1 and 2 are isomeric dipalmitate derivatives analysing by GLC as containing two fat molecules relative to Z-Ala-TRIS (56% and 59% resp.; Theory 59.6%) while Fraction 3 was consistent with being a monopalmitate derivative (fat content was 40%; Theory 42.5%). The yield by weight of Fraction 3 was 208 mg (24.5% Theory).

EXAMPLE 19BOC-Ala-Ile-Phe-TRIS-TRIPALMITATE

BOC-Ala-Ile-Phe-TRIS-tripalmitate was prepared in a similar manner to Example 12 from a solution of BOC-Ala-Ile-Phe-TRIS (500 mg in 30 ml chloroform; 5 ml DMF); palmitic acid (1.1 g in 20 ml chloroform), 825 mg of DCC in 10 ml chloroform and DMAP (40 mg in 10 ml chloroform) at 0°C for 2 hours and at room temperature for 16 hours.

After the removal of DCU, the chloroform solution was washed as previously described (water, citric acid, water, sodium hydrogen carbonate, water). After the removal of solvent, the oily residue was dried (Crude yield 1.66 g).

The crude product was dissolved in hexane and a portion of the solution purified on a Florisil column. Hexane: ether solvent mixtures removed the unreacted

- 20 -

palmitic acid and other impurities. The title product was then eluted with chloroform:methanol 50:50 (150 ml). The product was obtained as an oil, which was lyophilised from t-butylalcohol. The white fluffy compound (770 mg; 66.4% Theory) gave a single spot on TLC; Rf 0.44 (solvent g) and 0.13 (solvent e). Anal. chem. for  $C_{74}H_{135}N_4O_{11}$  : C70.71, H10.83, N4.46. Found C70.72, H10.78, N4.14. Fat content by GLC was 58% (Theory 56.9%).

#### 10 EXAMPLE 20

##### Z-Ala-ETHANOLAMINE-MONOPALMITATE

540 mg of the Z-Ala-ethanolamine was reacted with 670 mg palmitic acid, 540 mg DCC and 32 mg of DMAP in DCM and DMF as per Example 12. The mixture was evaporated to dryness and purified on Florisil. 219 mg of product (20% theory) was isolated. TLC Rf 0.16 (solvent g), 0.14 (solvent c, twice) and 0.12 (solvent d).

#### EXAMPLE 21

##### MITOGENIC EFFECTS OF Z-Ala-TRIS-TRIPALMITATE AND Ala-TRIS-TRIPALMITATE

Lipopeptides of the type Pam<sub>3</sub>-Cys-Ser-(Lys)<sub>4</sub> and Pam<sub>3</sub>-Cys-Ala-Gly are potent lymphocyte and macrophage activators (e.g. Reitermann et al., Biol. Chem. Hoppe-Seyler, 370, pp343-352). These compounds have also been shown to induce tumour cytotoxicity (Hoffmann et al 1989. Biol. Chem. Hoppe-Seyler, 370, pp575-582) in murine bone marrow derived macrophages.

The mitogenic effects of two of our constructs, Z-Ala-TRIS-Tripalmitate and Ala-TRIS-Tripalmitate were examined as follows:

##### INCORPORATION OF <sup>3</sup>H-THYMIDINE

<sup>3</sup>H-thymidine incorporation experiments were carried out in Linbro-Titertek microtitre plates (Flow Laboratories, Inc., U.S.A.) following the method of L. Bradley in "Selected Methods of Cellular Immunology (1980)

- 21 -

Eds. B.B. Mishell and S.M. Shiigi; Publisher. W.H. Freeman and Co., U.S.A. pp156. The tests were carried out externally by Dr. D. Rathjen of Peptide Technology Ltd., Dee Why, N.S.W.

5 Cell lines tested were:-

1. RD10; a B-cell lymphoma (obtained from the Centre for Immunology, Sydney University, Sydney, NSW.
  2. U937; a human monocyte-like<sup>3</sup> histiocytic lymphoma (source: American Tissue Culture Collection).
- 10

#### METHOD (EXAMPLE)

RD10 cells (at a cell density  $5 \times 10^5$ /ml, 100 ul/well) were cultured for 20 hours at 37°C with 5% CO<sub>2</sub> in RPMI 1640 medium, supplemented with 10% Fetal Calf. Serum. Samples to be tested were mixed and sonicated with this medium at a concentration of 5000, 500.50 and 5 ug/ml and 100 ul aliquots added to the cells. The cells were pulsed with 0.5 uCi/well of <sup>3</sup>H-thymidine and the plates incubated for four more hours before harvesting (Titertek Skatron harvester, Lier, Norway). Incorporated radioactivity was measured by liquid scintillation counting. All assays were performed in triplicate.

15

20

#### 25 RESULTS

Figures 6 and 7 summarize the results.

<sup>3</sup>H-thymidine uptake by RD10 cells showed a 3-4 fold increase in cell proliferation compared to control. This is in the range of other known mitogenic compounds e.g., phytohaemagglutinin gives a 5-10 fold increase under these conditions. The samples containing Ala-TRIS-Tripalmitate, where the N-terminal of the Alanine is free, showed a slightly diminished response compared with Z-Ala-TRIS-Tripalmitate.

30

35 Thymidine incorporation of the U937 cells was, in

- 22 -

slight contrast, close to control, and at higher concentrations indicated slight cytotoxic effects. As in the experiments using B-cells, Ala-TRIS-Tripalmitate samples showed slightly diminished responses compared with

5 Z-Ala-TRIS-Tripalmitate.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the

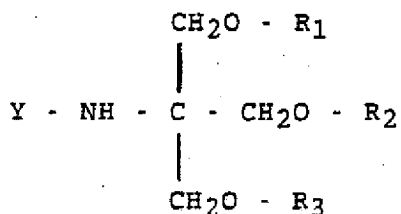
10 invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.



- 23 -

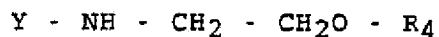
## CLAIMS:-

1. A compound of the following formula:-



in which Y represents an amino acid, peptide or derivative thereof and  $\text{R}_1$ ,  $\text{R}_2$  and  $\text{R}_3$  are the same or different and are either hydrogen or fatty acids.

2. A compound as claimed in claim 1 in which each of  $\text{R}_1$ ,  $\text{R}_2$  and  $\text{R}_3$  are fatty acids.
3. A compound as claimed in claim 2 in which  $\text{R}_1$ ,  $\text{R}_2$  and  $\text{R}_3$  are the same fatty acid.
4. A compound as claimed in any one of claims 1 to 3 in which the fatty acid has a carbon chain of 3 to 18 carbon atoms.
5. A compound as claimed in claim 4 in which the fatty acid has a carbon chain of 10 to 18 carbon atoms.
6. A compound as claimed in claim 5 in which the fatty acid has 16 carbon atoms.
7. A compound as claimed in any one of claims 1 to 7 in which Y is cysteine.
8. A compound of the following formula:-



in which Y represents an amino acid, peptide or derivative thereof and  $\text{R}_4$  represents hydrogen or a fatty acid.

9. A compound as claimed in claim 8 in which  $\text{R}_4$  is a fatty acid having a carbon chain of 3 to 18 carbon atoms.

- 24 -

10. A compound as claimed in claim 9 in which the fatty acid has a carbon chain of 10 to 18 carbon atoms.
11. A compound as claimed in claim 10 in which the fatty acid has 16 carbon atoms.
12. A compound as claimed in any one of claims 8 to 11 in which Y is cysteine.

1/7

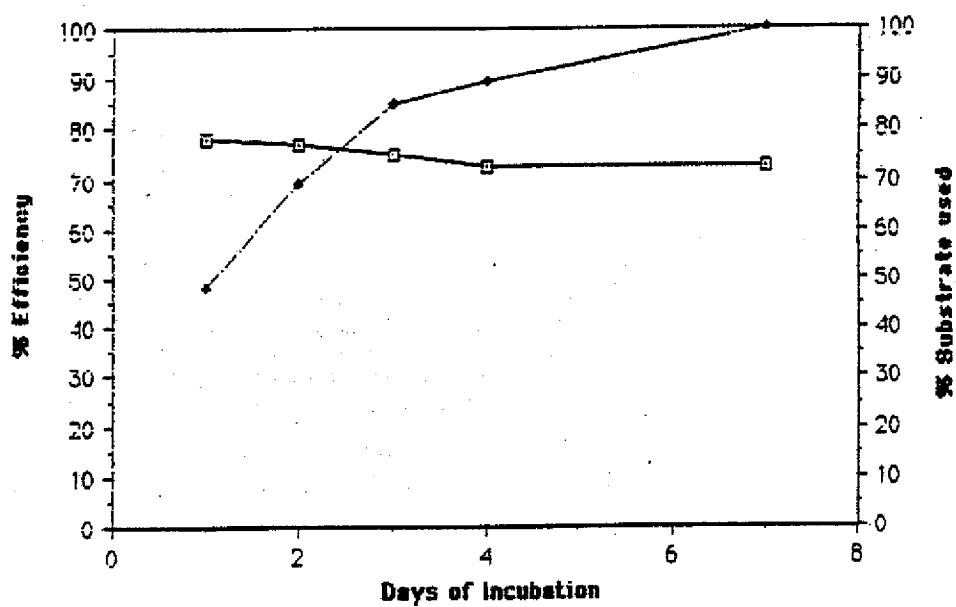


FIGURE 1.

2/7

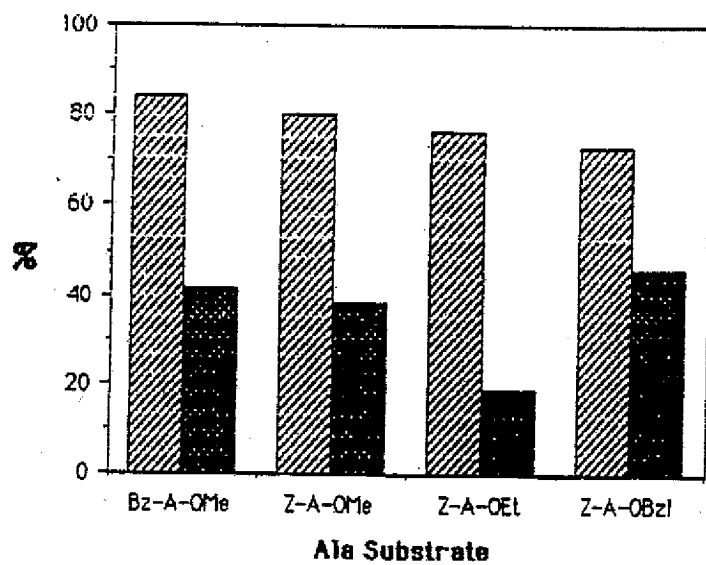


FIGURE 2.

3/7

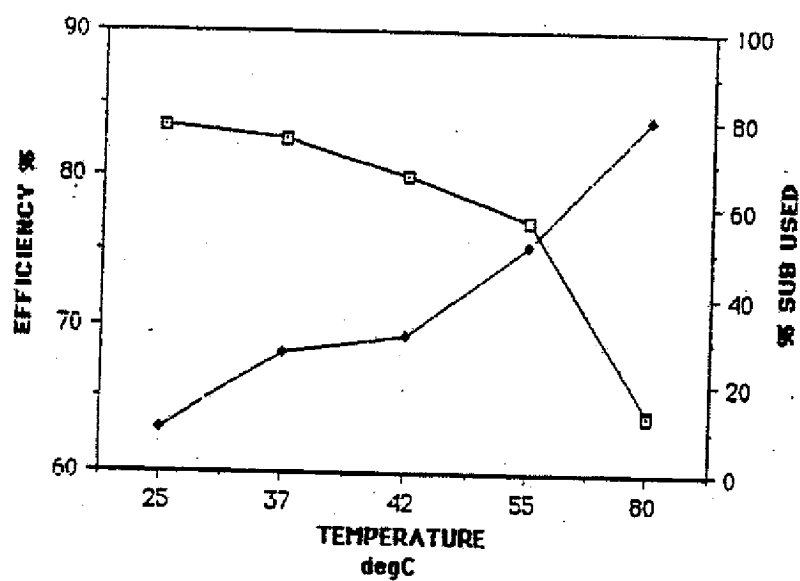


FIGURE 3.

4/7

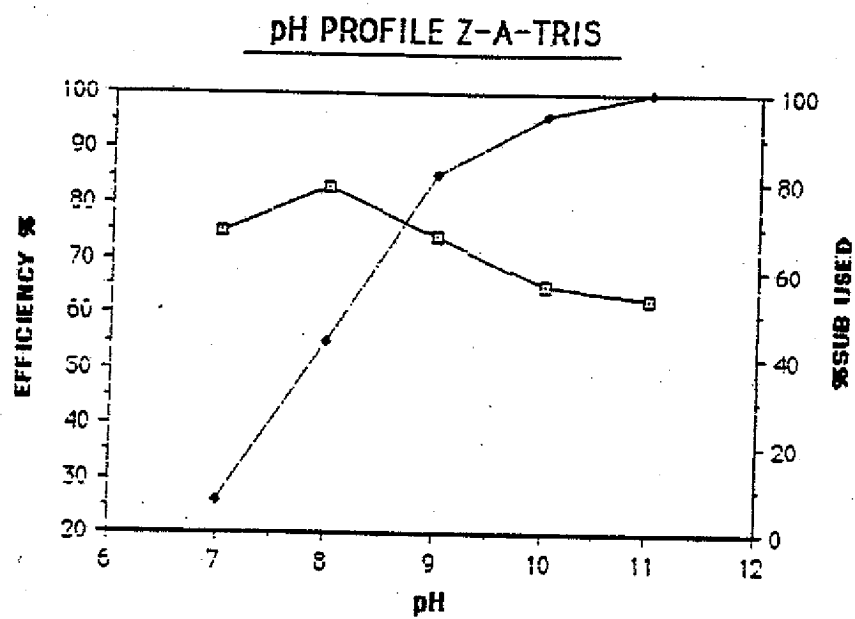


FIGURE 4.

5/7

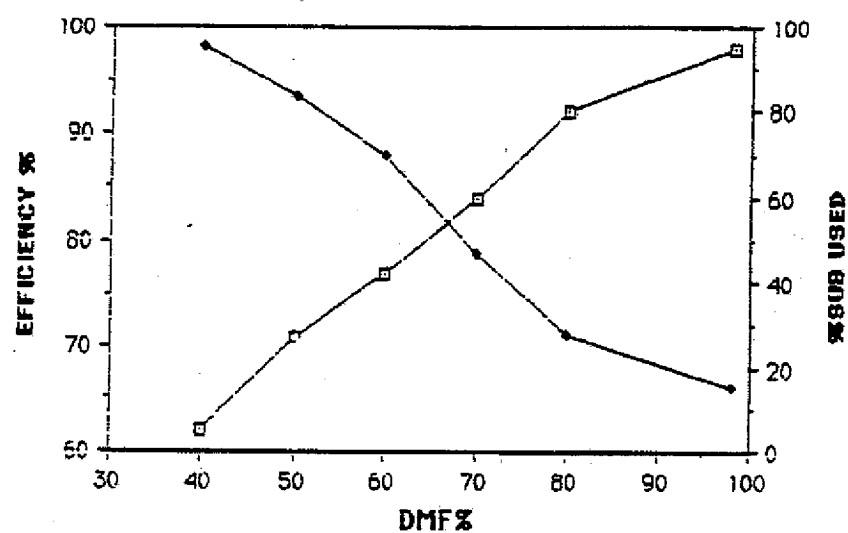


FIGURE 5.

6/7

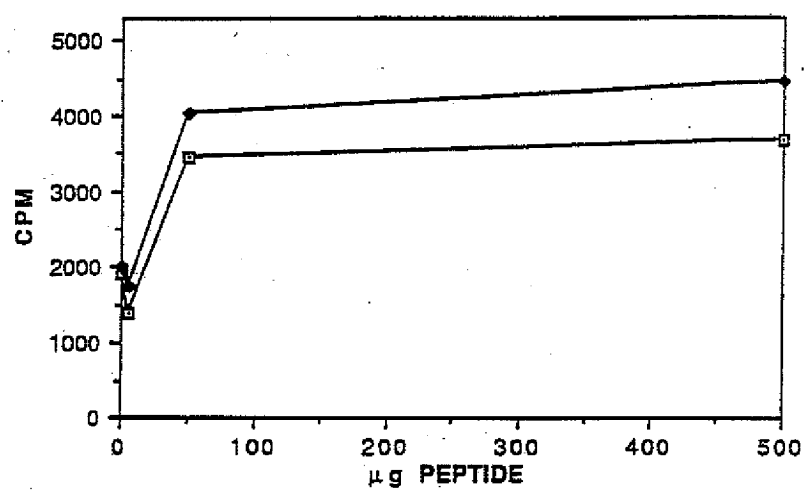


FIGURE 6.



7/7

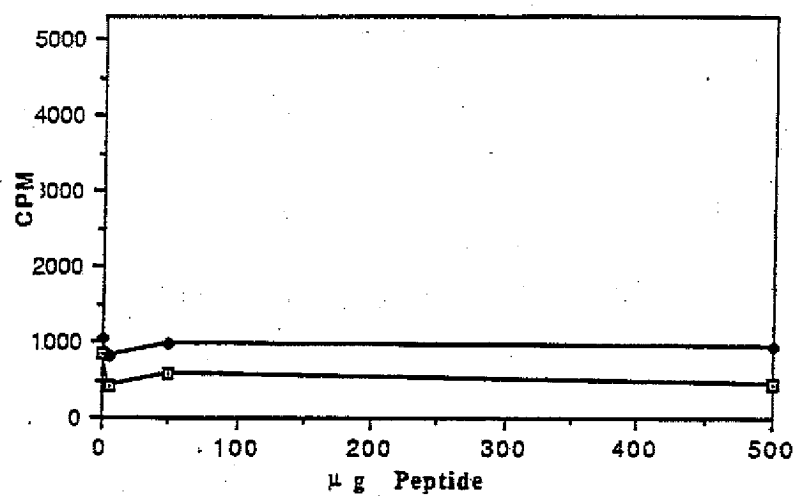


FIGURE 7.

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/AU 90/00599**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) 6				
According to International Patent Classification (IPC) or to both National Classification and IPC				
Int. Cl. <sup>5</sup> C07C 271/22; C07C 323/60; C07K 5/06; 5/08				
<b>II. FIELDS SEARCHED</b>				
Minimum Documentation Searched 7				
Classification System	Classification Symbols			
IC <sup>3</sup>	C07C 271/22; 323/60; C07K 005/06; 005/08			
IC <sup>4</sup>	C07C 125/065; 149/43; C07K 005/06; 005/08			
IC <sup>3</sup>	C07C 125/063; 149/43; 103/52			
IC <sup>2</sup> IC <sup>1</sup>	C07C 125/06; 149/43; 149/42			
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8				
AU: IPC as above Chemical Abstracts: using molecular formulae C <sub>7</sub> H <sub>15</sub> NO <sub>5</sub> and C <sub>7</sub> H <sub>15</sub> NO <sub>5</sub>				
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> 9				
Category*	Citation of Document, with indication <sup>11</sup> where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No 13		
A	Patent Abstracts of Japan, C-78, page 378, JP,A, 53-15325 (FUJISAWA YAKUHIIN KOGYO K K) 13 February 1978 (13.02.78)	1-12		
A	WO,A, 85/04398 (TAKEDA CHEMICAL INDUSTRIES, K K) 10 October 1985 (10.10.85)	1-12		
A	WO,A, 86/04894 (TAKEDA CHEMICAL INDUSTRIES, K K) 28 August 1986 (28.08.86)	1-12		
A	AU,A, 28694/89 (HOECHST AKTIENGESELLSCHAFT) 27 July 1989 (27.07.89)	1-12		
A	GB,A, 2217319 (GIBBONS W A) 25 OCTOBER 1989 (25.10.89)	1-12		
<p>* Special categories of cited documents: 10</p> <table style="width: 100%;"> <tr> <td style="width: 50%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%;"> <p>"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p> </td> </tr> </table>			<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>
<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>			
<b>IV. CERTIFICATION</b>				
Date of the Actual Completion of the International Search 8 April 1991 (08.04.91)	Date of Mailing of this International Search Report 11 April 1991			
International Searching Authority  Australian Patent Office	Signature of Authorized Officer  J H CHAN <i>[Signature]</i>			

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim numbers ..., because they relate to parts of the international application that do comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No